1999

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Vet Pathol **36**:406–411 (1999)

Demonstration of *Tritrichomonas foetus* in the External Genitalia and of Specific Antibodies in Preputial Secretions of Naturally Infected Bulls

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Abstract. Portions of penis and prepuce were collected from 24 bulls with current or recent *Tritrichomonas foetus* infection. Epididymides were collected from seven of the bulls, and seminal vesicles and prostate were collected from four. Following immunohistochemical staining with two monoclonal antibodies (34.7C4.4 and TF1.15) prepared against *T. foetus* surface antigens, trichomonads were identified in sections from 15 of the bulls. Organisms were most often located in penile crypts in the midshaft and caudal regions and less often in preputial crypts. Trichomonads were not observed in sections from other genitalia or in subepithelial tissue. *T. foetus* antigen, however, was present in the cytoplasm of some epithelial cells and the cytoplasm of some mononuclear cells in subepithelial lymphoid aggregates and follicles. Preputial smegma was collected from 16 *T. foetus*-infected bulls and from 16 control bulls with negative *T. foetus* cultures. Preputial antibody levels to TF1.17, a surface antigen of *T. foetus,* were determined by an enzyme-linked immunosorbent assay. Preputial secretions from infected bulls contained specific antibody of each isotype and subisotype tested. IgG1 responses were the greatest, IgM and IgA responses were approximately equal, and IgG2 responses were low. Each isotype and subisotype response in infected bulls was significantly greater than that in the controls. These results confirm previous speculation concerning anatomical sites of infection and suggest that parasite antigen can be taken up and processed locally, resulting in deposition of specific IgG1, IgG2, IgA, and IgM antibodies in the preputial cavity.

Key words: Cattle; genitalia; immunity; immunoglobulins; immunohistology; trichomoniasis; *Tritrichomonas foetus.*

Bovine trichomoniasis, caused by the protozoa *Tritrichomonas foetus,* is a venereally transmitted infection of cattle that produces early pregnancy loss, occasional pyometra, and, less commonly, mid- to late-term abortions. Bulls maintain the infection in the preputial cavity and may experience mild transient balanoposthitis.[22] Although young bulls can become infected, infection rates increase with age,[13,32] and evidence suggests that bulls ≥4 years of age become persistently infected.[23] *T. foetus* can be cultured from all regions of the penile and preputial mucosae and occasionally from the urethral orifice.[25] Highest numbers of organisms have been found on the penile mucosa and adjacent posterior preputial mucosa.[18]

In a previous histopathologic study of naturally and experimentally infected bulls, no lesions could be specifically attributable to *T. foetus* infection.[25] No organisms were detected in tissue sections by conventional stains or fluorescence microscopy using acridine orange and an immunofluorescent technique that employed a polyclonal antibody against *T. foetus. T. foetus* was considered limited to surface secretions in the preputial cavity. In heifers and cows, the organism is found on the endometrial surface and in endometrial glandular lumina.[1,26]

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Invasion of placental chorion and fetal tissues by *T. foetus* has been demonstrated using conventional stains and immunohistochemical (IHC) techniques.[27,28] Tissue invasion by other species of trichomonads also has been reported,[7,31] including prostatic[16] and cervical[17] invasion by *Trichomonas vaginalis* in human beings. This variation in the invasiveness of trichomonads warranted a more detailed study on the location of the organism in the genital tissues of bulls. The bull's local immune response to *T. foetus* infection is poorly understood. Systemic immunization of bulls with trichomonad antigen can prevent or cure infection,[11,12] yet unvaccinated bulls often remain persistently infected. The purposes of this study were to immunohistologically identify the anatomical sites of *T. foetus* infection in the bull's genitalia, to determine if *T. foetus* antigen crosses the epithelium, and to quantify the local (intrapreputial) antibody responses, by isotype, to the parasite.

Materials and Methods

Genital tissues from nine bulls from Saskatchewan (Canada) (bull Nos. 1–9), 14 bulls from California (bull Nos. 10–23), and one bull from New Mexico (bull No. 24) were used in this study. All bulls were beef breeds, and all were from *T. foetus*-infected herds. Ages of the bulls ranged from 2 to 6 years. All of the bulls except Nos. 3, 5, 8, and 9 were culture positive for *T. foetus* at slaughter; Nos. 3, 5, 8, and 9 had been culture positive when tested within 4 months prior to slaughter but were culture negative at slaughter.

Specimens collected at slaughter included portions of the galea glandis, glans penis, shaft of the penis midway between the glans and fornix, prepuce cranial to the fornix, and prepuce just inside the preputial orifice. In addition, portions of the epididymis were collected from bull Nos. 1–3, 7–9, and 24, and seminal vesicles and prostate were collected from bull Nos. 7, 8, 9, and 24.

Tissues were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. IHC staining was done on adjacent sections using a modified technique[29] that utilizes a commercially available labeled streptavidin–biotin system (Dako, Carpinteria, CA). Paraffin-embedded sections 4 μm thick were mounted on positively charged slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA), deparaffinized, hydrated to buffer (pH 7.5), treated with 3% H_2O_2 for 5 minutes to quench endogenous peroxidase, enzyme digested for 6 minutes at 37 C (Proteinase K, Dako), and incubated with one of two monoclonal antibodies (MAbs) developed against *T. foetus* for 20 minutes at 37 C. Adjacent sections were treated with the two MAbs, 34.7C4.4[8] and TF1.15.[19] These MAbs were chosen because each recognizes a surface antigen of *T. foetus.* Each MAb was diluted 1 : 500 with Tris buffer. After three rinses in buffer (10× automation buffer, Biomeda, Foster City, CA), sections were incubated with biotinylated goat anti-mouse and anti-rabbit Ig for 10 minutes at 37 C. Following three additional rinses in buffer, peroxidase-labeled streptavidin was applied for 10 minutes at 37 C. Sections were then rinsed, and the enzyme activity was detected using 3% 3-amino-9-ethylcarbozole in *N,N*-dimethylformamide (Dako). Sections were counterstained with Gill II hematoxylin (Surgipath Medical Industries, Grayslake, IL) for 3 minutes, rinsed in water, and blued in buffer for 1 minute. Coverslips were mounted with an aqueous mounting medium (Dako). Nonimmunized rabbit Ig was substituted for primary antibody as a negative control. A commercially available capillary gap system (MicroProbe, Fisher Scientific) was used for all IHC staining.

Preputial smegma was collected for antibody analysis from the 14 culture-positive bulls from California plus two more from the same study (16 total). Smegma was also collected from 16 control bulls with negative *T. foetus* cultures. Positive bulls were cultured many times, but negative bulls were only cultured once as part of a previously reported prevalence study.[4] Because three cultures are recommended to be sure that a bull is negative for *T. foetus,*[3] some of the negative control bulls may have been false negatives. The samples were collected using a 20-in. dry plastic uterine infusion pipette attached to a 12-ml disposable plastic syringe. The pipette was introduced into the preputial cavity up to the fornix. Several vigorous back and forth movements with simultaneous negative pressure from the syringe were made to collect each sample. The pipette was removed from the prepuce and the pipette tip was rinsed in 1 ml of phosphate-buffered saline (PBS), pH 7.2, or lactated Ringer's solution. Samples were frozen at −20 C until used.

Smegma samples were tested for IgG1, IgG2, IgM, and IgA antibodies to the TF1.17 surface antigen of *T. foetus* by enzyme-linked immunosorbent assay (ELISA) as previously described[21] with modifications. (MAbs TF1.15 and TF1.17 recognize different epitopes of the same antigen.[20]) The TF1.17 antigen was prepared by immunoaffinity purification as previously described.[5] Half-well microtiter plates (Costar, Cambridge, MA) were coated with 50 μl/well of TF1.17 antigen at a protein concentration of 3 μg/ml in 0.1 M carbonate coating buffer, pH 9.6, and incubated overnight at room temperature. The next morning the wells were forcibly emptied, and each was filled with 50 μl of 95% ethanol for 2 minutes. The wells were forcibly emptied again, refilled with ethanol, and incubated for 3 hours at room temperature in a fumehood followed by another 1 hour at 37 C. The plates were blocked overnight with 150 μl/well of PBS containing 0.02% sodium azide (Sigma Chemical, St. Louis, MO) and 3% gelatin (DIFCO Laboratories, Detroit, MI) in a moist chamber at 37 C. Wells were washed three times with 150 μl/well of PBS containing 0.05% Tween 20 (PBS-T, Sigma). Thawed preputial secretions were centrifuged at 17,500 × *g* for 5 minutes, and the supernatants were diluted with an equal volume of PBS-T containing 0.3% gelatin (PBS-T-gel, Sigma). Wells were filled with 50 μl of the diluted samples and incubated for 1 hour at 37 C. After washing three times with PBS-T, wells were incubated for 30 minutes at 37 C with 50 μl of mouse MAbs to bovine IgA, IgG1, IgG2, and IgM (DAS 7, 16, 2, or 11 provided by A. Guidry, US Department of Agriculture, Beltsville, MD) diluted in PBS-T-gel at 1 : 4,000; 1 : 8,000; 1 : 2,000, and 1 : 8,000, respectively. Wells were again washed three times and filled with 50 μl peroxidase-conjugated rabbit anti-mouse

IgG (Zymed Laboratories, South San Francisco, CA) diluted 1 : 2,000 and incubated at 37 C for 30 minutes. The final three washes were done before filling with 50 μl of TMB/hydrogen peroxide substrate (Kirkegard and Perry Laboratories, Gaithersberg, MD) and incubating at room temperature in the dark for 30 minutes. Stop reagent (0.3 M phosphoric acid) was added (50 μl/well) and absorbance was read at dual wavelengths (450 and 650 nm) on an enzyme-linked immunosorbent assay (ELISA) microplate reader (Molecular Devices, Menlo Park, CA). Negative control wells included antigen but no preputial secretions, and positive control wells contained convalescent serum or vaginal mucus known to have anti-TF1.17 antibodies. Each individual sample was analyzed in duplicate, with mean and standard error reported. For the culture-positive bulls, two samples collected about 6 weeks apart were analyzed in duplicate, with means and standard error reported. The protein content of each was determined by the Bradford method[6] as performed according to the manufacturer's instructions (BioRad Laboratories, Hercules, CA). Only samples with >0.2 mg/ml of protein were included in the analysis.

Differences in ELISA absorbances for preputial secretions of infected or control bulls were analyzed by a one-tailed *t*-test.

Results

The IHC technique labeled trichomonads in sections from four of the nine Canadian bulls, 10 of the 14 California bulls, and the New Mexico bull. Two of the Canadian bulls found positive by the IHC technique were culture positive at the time of slaughter (Nos. 1, 7), and two bulls (Nos. 5, 8) had been culture positive within the previous 4 months but were negative at slaughter.

The MAb TF1.15 more consistently labeled trichomonads than did MAb 34.7C4.4. Both antibodies used at the 1 : 500 dilution gave little or no background staining. One unique feature of TF1.15 was that, in addition to labeling trichomonads, it strongly labeled peripheral nerve myelin sheaths. Trichomonads were most often located in penile crypts in the midshaft and caudal regions (14 bulls) and less often in preputial crypts (five bulls). The organisms were typically located in deposits of cellular detritus (smegma) in a few selected crypts (Fig. 1). Only one or two crypts in a section contained organisms, but the same crypts contained clusters of trichomonads in several serial sections from the block. Intact trichomonads were occasionally present two or three cell layers deep in the stratified squamous epithelium of the crypts but were never observed subjacent to the basement membrane. Trichomonads were not seen in the penile or prostatic urethra or in sections of seminal vesicles, prostate, or epididymides.

Inflammatory cell infiltrates were present in the penis and prepuce of all infected bulls and consisted of subepithelial lymphoid nodules with overlying epithelium containing marked lymphoid infiltration, diffuse subepithelial lymphoplasmacytic infiltrates, focal subepithelial and intraepithelial accumulations of neutrophils, and subepithelial perivascular accumulations of lymphocytes, plasma cells, and occasionally eosinophils. Small numbers of neutrophils were occasionally mixed with cellular detritus in penile and preputial crypts. Trichomonads were typically located in crypts lacking inflammatory changes but were also present in some crypts containing neutrophils. Rarely, whole trichomonads were present in the cytoplasm of phagocytes in crypts.

There was diffuse IHC staining of the cytoplasm and/or nucleus of some epithelial cells adjacent to trichomonads, and small granular staining was often present in the cellular detritus surrounding organisms (Figs. 2, 3). In addition, large, active lymphoid follicles with marked mononuclear cell infiltration of the overlying epithelium were present in proximity to some clusters of trichomonads. Fine granules and larger globules of IHC-positive antigen were present in the cytoplasm of rare mononuclear phagocytes located in subepithelial lymphoid nodules or in diffuse subepithelial mononuclear cell infiltrates (Fig. 4). There was no labeling of trichomonads or myelin sheaths in negative control sections treated with nonimmune serum.

Preputial secretions of infected bulls contained anti-TF1.17 antibody of each isotype and subisotype tested (Fig. 5). IgG1 responses were greatest, IgA and IgM responses were approximately equal, and IgG2 responses were very low. In each case, the response in infected bulls was significantly greater than that in the controls (IgG1, $P = 0.01$; IgG2, $P = 0.04$; IgM, $P = 0.04$; IgA, $P = 0.03$).

Discussion

Using the IHC technique, *T. foetus* organisms could be demonstrated in penile crypts of the majority of infected bulls examined. The penile and preputial crypt location is identical to that of *Campylobacter fetus,*[30] another motile pathogen that prefers low oxygen tension. Although organisms were present in the superficial layers of epithelium, evidence of invasion of the basement membrane and dermis by intact *T. foetus* was not observed. Trichomonads were not observed in the urethra, epididymis, or accessory sex glands; however, only a small number of these specimens were available for study. The tendency of the organism to colonize only occasional crypts suggests that punch biopsy of penile or preputial epithelium as a method of diagnosis might lack sensitivity.

An unexpected finding was the strong labeling of nerve myelin sheaths by the MAb TF1.15. Previous characterization of the antigen labeled by TF1.15 has shown it to be highly glycosylated[19] with a glycolipid moiety (B. N. Singh, personal communication). Gly-

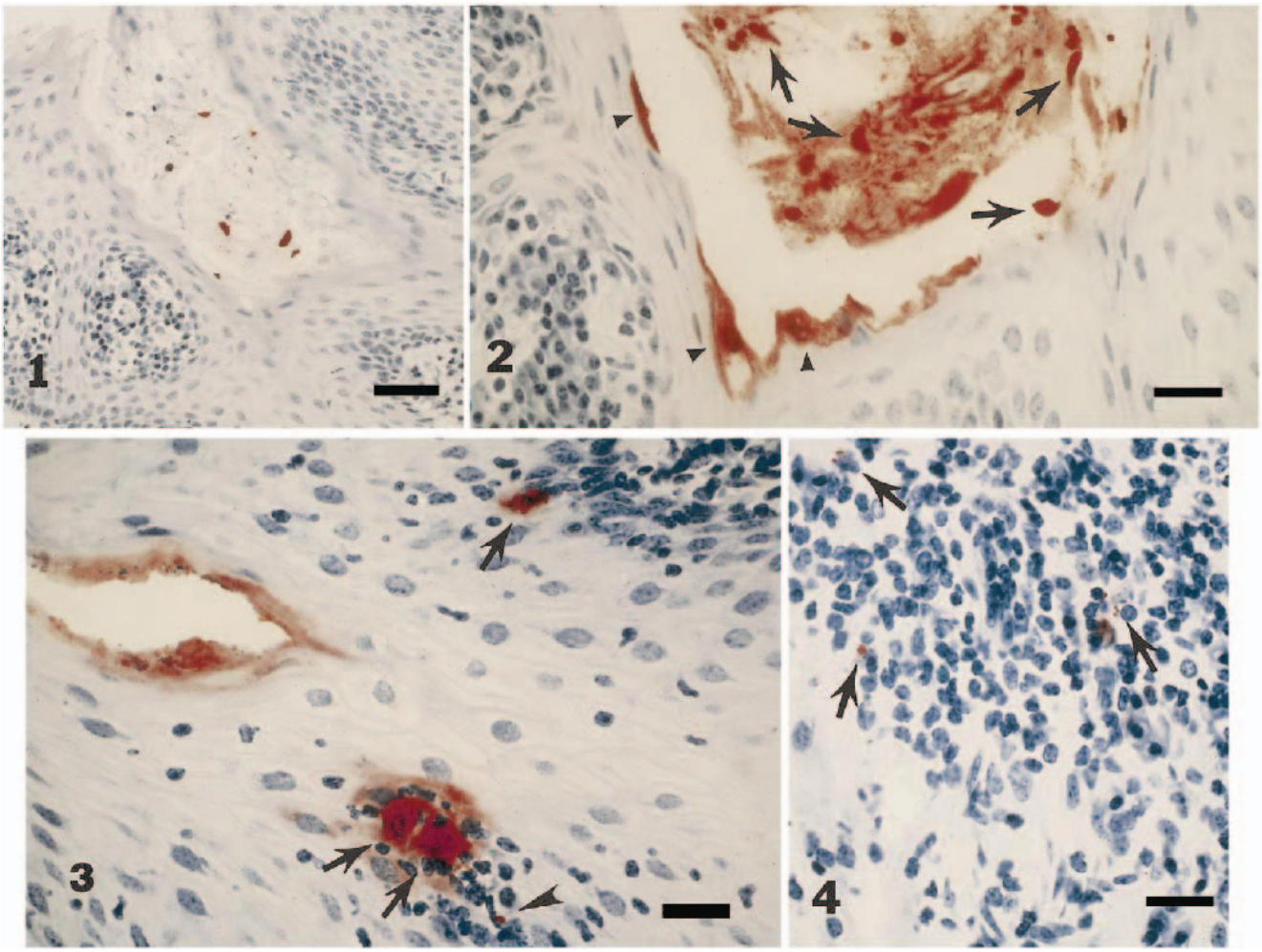

Fig. 1. Penis; bull No. 13. Immunolabeled (red) trichomonads are located in detritus in penile crypt. MAb 34.7C4.4 in labeled streptavidin–biotin–peroxidase method, Gill II hematoxylin counterstain. Bar = 40 μm.

Fig. 2. Penis; bull No. 13. Note diffuse granular labeling of detritus, identifiable trichomonads (arrows), and diffuse staining of few superficial crypt epithelial cells (arrowheads). MAb 34.7C4.4 in labeled streptavidin–biotin–peroxidase method, Gill II hematoxylin counterstain. Bar = 20 μm.

Fig. 3. Penis; bull No. 13. There is marked diffuse cytoplasmic staining of few cells (arrows) near the basilar layer of crypt epithelium and focal labeled antigen in the cytoplasm of phagocyte (arrowhead) located subjacent to labeled epithelial cells. MAb TF1.15 in labeled streptavidin–biotin–peroxidase method, Gill II hematoxylin counterstain. Bar = 20 μm.

Fig. 4. Penis; bull No. 24. Note focal labeling in cytoplasm of few mononuclear cells (arrows) in subepithelial lymphoid nodule. MAb TF1.15 in labeled streptavidin–biotin–peroxidase method, Gill II hematoxylin counterstain. Bar = 15 μm.

colipids are important constituents of myelin with apparent cross-reactivity to the *T. foetus* surface antigen recognized by TF1.15. MAb TF1.15 labeled trichomonads more consistently than did MAb 34.7C4.4; this finding is consistent with previous work showing that the antigen labeled by MAb TF1.15 is widely conserved in *T. foetus* strains.[20] A previous IHC study[29] evaluating MAb 34.7C4.4 demonstrated that the antibody specifically labeled *T. foetus* and only faintly labeled one other trichomonad, *Trichomonas gallinae.* Other protozoa, bacteria, and fungi were not labeled. In the current study, both antibodies labeled only trichomonads, cells and detritus in proximity to trichomonads, and peripheral nerve myelin (TF1.15). The staining of cells in proximity to *T. foetus* demonstrates antigen uptake by the cells, and the few stained cells below the epithelium in this area are likely to be antigen-presenting cells. The staining of peripheral nerve myelin was of equal intensity to trichomonad staining, suggesting a specific antibody reaction with cross-reactive epitopes.

Histologic findings in these bulls were consistent with those described previously[15,25] and were not considered specific for *T. foetus* infection. Subepithelial infiltrates of plasma cells and focal accumulation of lymphocytes and macrophages have been detected pre-

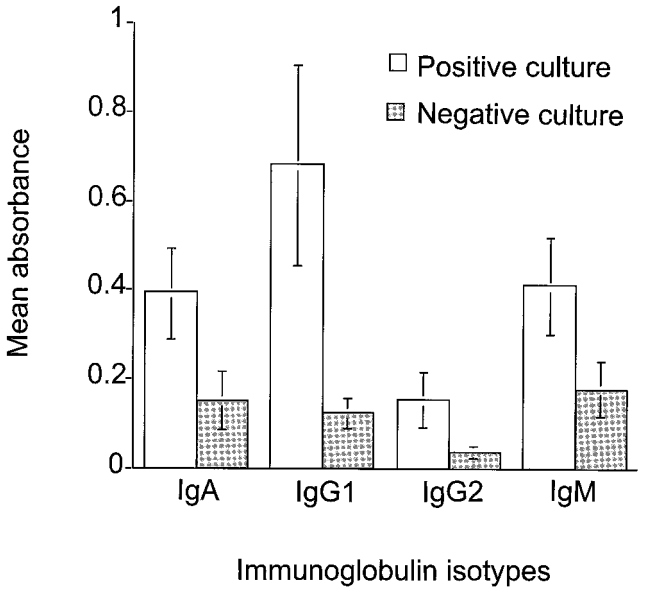

Fig. 5. Isotypic antibody responses to TF1.17 antigen in preputial secretions of 16 infected and 16 control bulls as determined by ELISA. Bars = mean of duplicate samples ± standard errors.

viously in the preputial and penile mucosa of bulls with campylobacteriosis[30] and trichomoniasis.[25] Even in normal mature bulls, the lamina propria of the preputial and penile mucosa is infiltrated with plasma cells and other mononuclear cells.[24,30] In a study of 83 bulls,[15] slightly higher numbers of plasma cells were found in the preputial lamina propria of bulls infected with *C. fetus* or *T. foetus* than in normal bulls. Also observed were lymphoid follicles associated with marked lymphoid infiltration of the overlying epithelium. This ''lymphoepithelium'' was similar to that found in tonsils. In the *T. foetus*-infected bulls in the present study, epithelial cells and a few mononuclear cells in subepithelial lymphoid follicles and mononuclear cell infiltrates stained with MAbs to *T. foetus* antigen, indicating that antigen was taken up by epithelial cells and transmitted to cells below the basement membrane. Because some mononuclear cells in lymphoid follicles are likely to be antigen-presenting cells, the components for inductive sites appear to be present in the lymphoid follicle/lymphoepithelium of the penile and preputial mucosae. The presence of plasma cells in the lamina propria suggests that antibody synthesis and secretion is taking place here also. Thus, the penile and preputial mucosae appear to be both inductive and effector sites.

Immunoglobulins and antibodies found in preputial secretions also suggest that antibody is synthesized in the lamina propria and transported across the epithelium. In previous studies, IgG was generally the most plentiful isotype in preputial secretions, followed by IgA and then IgM.[2,15] IgG2 predominated over IgG1 in older bulls. In neither of these studies were specific antibodies of each isotype measured. Although antibodies were not detected in preputial washings of trichomonal-infected bulls in earlier studies,[10,33] the preputial secretions may have been more dilute and the detection techniques not as sensitive as those in the present study. High levels of IgG1 antibody to *T. foetus* surface antigen and specific IgA and IgM antibodies were found in the present study, suggesting that the lymphoid follicles under the lymphoepithelium were inductive sites and that the plasma cells in the lamina propria produced these antibodies locally. IgG1 often predominates in bovine secretions such as milk, intestinal contents, and tears, and local synthesis and selective transport have been proposed to explain its presence.[9] As in other species, IgA is known to be an important secretory immunoglobulin. The relatively high levels of IgM and low IgG2 antibody to TF1.17 antigen in the present study may have been due to the nature of the antigen. TF1.17 is highly glycosylated and monoclonal and polyclonal antibodies to TF1.17 are often IgM.[19] Furthermore, heifers immunized with TF1.17 antigen produce high levels of serum and vaginal IgG1 but much lower levels of IgG2 antibodies.[5,14] This tendency of TF1.17 antigen to stimulate IgM and IgG1 antibodies may explain the low levels of IgG2 specific antibody.

In a previous study, total immunoglobulin levels in preputial washings were greater in normal bulls than in bulls infected with *T. foetus,*[15] possibly because of digestion of Ig by the extracellular proteinase of *T. foetus.*[34,35] The greater levels of IgG1, IgA, IgM, and IgG2 specific antibodies to *T. foetus* antigen (as opposed to the total immunoglobulins) in infected bulls than in control bulls indicates that local antibody responses to trichomonads do occur during infection. Furthermore, preputial antibody titers to *T. foetus* can be increased by systemic immunization and challenge,[10] and systemic immunization of bulls can be protective against trichomoniasis.[11,12]

IHC staining for specific antigens of *T. foetus* allowed identification of the anatomical sites of infection and strongly suggested that parasite antigen can be taken up and processed locally. These findings plus the ELISA results show that local antigen processing results in deposition of specific IgG1, IgG2, IgA, and IgM antibodies in preputial secretions. These results encourage further manipulation of the bull's immune response to enhance the effectiveness of immunologic protection at the local level.

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